

Transport of Pru p 3 across gastrointestinal epithelium – an essential step towards the induction of food allergy?

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Summary

Background Since intestinal absorption of food protein can trigger an allergic reaction, the effect of plant food allergen on intestinal epithelial cell permeability and its ability to cross the epithelial monolayer was evaluated.

Objective To study the interaction of Pru p 3 with intestinal epithelium, its natural entrance, analyzing transport kinetics and cellular responses that trigger.

Methods This was achieved using Pru p 3, the peach LTP, as a model. Enterocytic monolayers were established by culturing Caco 2 cells, as a model of enterocytes, on permeable supports that separate the apical and basal compartments. Pru p 3 was added to the apical compartment, the transepithelial resistance (TEER) was measured, and the transport was quantified.

Results The peach allergen that crossed the cell monolayer was detected in the cell fraction and in the basal medium by immunodetection with specific antibodies and the quantity was measured by ELISA assay. Pru p 3 was able to cross the monolayer without disturbing the integrity of the tight junctions. This transport was significantly higher than that of a non-allergenic peach LTP, LTP1, and occurred via lipid raft pathway. The incubation of Caco 2 cells with Pru p 3 and LTP1 produced the expression of epithelial-specific cytokines TSLP, IL33 and IL25.

Conclusion These results suggest that Pru p 3 was able to cross the cell monolayer by the transcellular route and then induce the production of Th2 cytokines. The results of the present study represent a step towards clarifying the importance of Pru p 3 as a sensitizer. **Clinical relevance** The capacity of food allergens to cross the intestinal monolayer could explain their high allergenic capacity and its fast diffusion through the body associating to severe symptoms.

Keywords allergenic LTPs, Caco 2 cell, epithelial transportation, food allergen, Pru p 3, Th2 cytokines

Introduction

The gastrointestinal tract is one of the largest immunologic organ in the body. Like all other mucosa-associated lymphoid tissues, the gut-associated lymphoid tissue (GALT) is in constant interaction with the external environment. Through a single epithelial layer and a complex immunologic network, the gut constantly deals with a large antigen load that can be beneficial or

harmful [1]. Epithelial cells play an essential role in the initiation and amplification of innate immune responses to viral infections [2]. Many diseases are exacerbated by, or may develop as a consequence of, loss of epithelial barrier function [3].

Allergies have become more prevalent in recent decades, and there is a pressing need to understand their underlying mechanisms. Sources of allergens, such as mites, cockroaches, fungi, and pollen, produce or

contain proteases. It has been proposed that proteolytic activity is involved in the pathogenesis of allergies, its possible effect by facilitating the passage of allergens across tissue barriers, cleaving molecules, and affecting the functions of various cells and immune responses [4, 5]. However, in plant allergens, transport across epithelia has been described without protease activity [6–9]. In the case of Bet v 1, it has been suggested that this allergen undergoes caveolae-mediated transport in the respiratory epithelium. Bet v 1 was detected in the interior of epithelial cells by staining patient biopsies. The transport was associated with cytoskeletal proteins, trafficking, and internalization [9]. The same was true for Phl p 1. In this case, Phl p 1 was detected inside A549 (an epithelium cell model) and induced a pro-inflammatory response with no monolayer alteration [10].

To mimic human intestinal epithelia, Caco 2 cells have been extensively used, mainly in drug transport studies. This cell line, which is derived from human colon adenocarcinoma [11], undergoes spontaneous enterocytic differentiation, leading to the formation of a monolayer of highly polarized cells, with tight junctions and microvilli on the apical membrane [12]. Several studies of food allergen transport have been carried out using this system in recent years [12–15]. Special permeable membranes, called Transwell® inserts, are used for these transport assays. The epithelial cells grow to confluence on these inserts and differentiate over the course of 3 weeks to give an enterocyte phenotype. The allergen is loaded in the apical side, and the basolateral medium is collected to quantify the amount transported. To test the integrity of the monolayer, transepithelial electrical resistance (TEER) is measured, to determine whether the tight junctions are correctly formed.

LTPs have been proposed as a model of true food allergens [16, 17], due to their capacity to sensitize by the oral route. Their high resistance to proteolytic digestion [18] and heat treatment [19, 20] suggests that they retain their immunogenic and allergenic motifs after passing through the gastrointestinal tract, and they can interact with the associated epithelial immune system to induce sensitization and systemic symptoms. Pru p 3, LTP and the major peach allergen, has been described as the primary sensitizer of the family of LTPs [16]. However, there are no studies that examine the interaction of this allergen with intestinal epithelium and the route followed. LTPs are associated with immunoglobulin E (IgE)-mediated food allergy and are a major cause of concern due to the severity of reactions they elicit. *In vitro* gastrointestinal digestion studies have shown LTPs to retain sufficient three-dimensional structure after extensive digestion [21], suggesting that it is possible for them to reach the gut immune system in an immunologically active form. In this study, we

have evaluated the absorption rates of Pru p 3 across human intestinal epithelial Caco 2 cell monolayers and made progress towards elucidating its transport mechanism. Pru p 3 was transported across the epithelial cells following a Michaelis–Menten curve, which would indicate the collaboration of a receptor transporter. This would be associated with cytoskeletal structures, especially desmosomes, with plakoglobin being the most plausible candidate. Moreover, Pru p 3 seemed to be associated to lipid rafts. In addition, epithelial cells responded to the presence of Pru p 3 with production of a typically Th2 cytokine profile.

Materials and methods

Cell culture

The Caco 2 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown following the manufacturer's instructions. Cells were seeded in 24-well Transwell® culture plates (0.4 mm pore diameter; Corning Inc., Corning, NY, USA) at a density of 8×10^4 cells per well and grown for 18–21 days, replacing the medium every 2 days. The integrity of the Caco 2 cell monolayer was checked by measuring the transepithelial electrical resistance (TEER) using a Millicell-ERS device (Millipore, Bedford, MA, USA). Cell monolayers were used in transport studies when values of TEER reached a plateau exceeding $300 \Omega\text{cm}^2$.

Allergen absorption experiments

Recombinant Pru p 3 (Pru p 3) and LTP1 (GenBank: EU424269) were isolated from cultured supernatants of transformed *Pichia pastoris* cells, as described by Diaz-Perales et al. [22]. For cell culture, the absence of LPS in the samples was checked by anti-LPS antibodies (rabbit anti-*E. coli* LPS; AbD Serotec, Kidlington, UK) and using THP1-XBlue cells (Invivogen, Toulouse, France).

Pru p 3 and LTP1 were labelled with Alexa 488 using an Alexa Fluor 488 Protein Labelling kit (Invitrogen, Carlsbad, CA, USA) added to the apical side of the Transwell® inserts at different concentrations in culture medium (Pru p 3: 15, 5, 3, 1 and 0.5 μg ; LTP1: 5 μg). After 2, 4, 8, and 24 h, basolateral samples were collected and an equal volume of culture medium was replaced immediately after each sampling in the basolateral chamber. At 24 h, apical samples were also recovered. The functionality of the cell monolayer was monitored by measuring TEER. Each experiment was repeated at least four times.

The transport of Pru p 3 and LTP1 was quantified by measuring the fluorescence of the supernatants

recovered from the basolateral or apical side, using a fluorescence microplate reader (Spectrafluor Fluorometer TECAN; TECAN group, Männedorf, Switzerland) at 485/535 nm. All tests were performed in four independent assays.

Simulated gastrointestinal digestion

Pru p 3 and LTP1 were submitted to simulated gastrointestinal digestion following the method described by Moreno et al. [23]. Pru p 3 and LTP1 were incubated with pepsin (1% w/vol; Calbiochem, La Jolla, CA, USA) in 100 mM HCl pH 2 simulated gastric fluid (SGF) at different times (0 min, without pepsin, 30 and 120 min after addition of pepsin). The reaction was stopped by adding 20 mM Tris-HCl pH 8.

In vitro duodenal digestion was performed by using 120-min gastric digestions as the starting material, adding a bile salt mixture [0.125 mM sodium taurocholate (Sigma, St. Louis, MO, USA), 0.125 mM glycodeoxycholic acid (Calbiochem)], 1 M CaCl₂ (BDH), 0.25 M Bistris, pH 6.5 (Sigma), porcine pancreatic lipase (0.1% w/v; Sigma) and porcine colipase (0.055%, w/v; Sigma). Finally, trypsin (0.1% w/v; Sigma) and α -chymotrypsin (0.4% w/v; Sigma) were added (1 allergen: 400 trypsin: 100 chymotrypsin). The digestion was performed at 37°C and was stopped after 120 min by heating at 90°C for 5 min.

Samples were analysed by SDS-PAGE.

Cocultures with PBMCs

Caco 2 cells were also cultured with peripheral blood mononuclear cells (PBMCs) from four healthy donors recruited from the Transfusion Centre (Madrid Health Service, Madrid, Spain) as previously described [24]. PBMCs were then seeded in the basolateral compartment of the Transwell® inserts (2×10^6 cells/well) in a volume of 600 μ L, and Pru p 3 (5 μ g) or LTP1 (5 μ g) was added in the apical chamber, as described above. The coculture was continued for 24 h, after which the apical and basolateral sides were recovered. The TEER was also measured at the end of the experiment to check the integrity of the monolayer. All the experiments were performed in four independent assays.

Immuno assays

Basolateral and apical supernatants, and cell fraction from Transwell® inserts, after 24 h of culture with 5 μ g of Pru p 3 were separated by SDS-PAGE on Bio-Rad Miniprotein III System gels (15% polyacrylamide) (Bio-Rad Laboratories, Hercules, CA, USA), according to the method of Laemmli and then electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. After

blocking, the membrane was incubated with a rabbit polyclonal antibody to Pru p 3 (1 : 1000 dilution; 1 h; kindly provided by Dr C. Pastor, Fundación Jiménez Díaz, Madrid, Spain), washed and incubated with goat anti-rabbit IgG-peroxidase conjugate antibody (1 : 10 000 dilution; 1 h) (Dako, Barcelona, Spain). Detection was achieved by means of enhanced chemiluminescence, according to the manufacturer's instructions (Amersham Biosciences, Little Chalfont, UK).

Specific IgE was determined by direct ELISA assay as previously reported [25, 26], using a pool of sera from 10 peach-allergic patients recruited at the Allergy Service, Fundación Jiménez Díaz (Madrid, Spain). Patients were selected on the basis of a convincing clinical history of allergic reactions after the ingestion of peach, a positive skin prick test (SPT) to Pru p 3 [27], and significant specific IgE levels to this allergen by ELISA assays. Plates were covered with rPru p 3 or LTP1 at different concentrations. Sera were diluted 1/2, and goat anti-human IgE-peroxidase conjugate antibody was used at 1 : 3000 dilution (Biosource, Camarillo, CA, USA). Blocking solution (Sigma) without solid phase was used as negative control, and OD values > 0.189 units ($n = 10$; mean $\pm 3 \times \text{SD} = 0.108 + 3 \times 0.026$ OD units) were considered positive. Serum dilution was determined by titration curves.

Basophil activation tests (BAT) were carried out with basolateral supernatants from Caco 2 cell cultures were incubated with a whole-blood sample from a peach-allergic donor, as described previously [28, 29]. A positive response was recorded when the stimulation index (antigen-specific response/basal level) was ≥ 2 .

Immunoprecipitation

Caco 2 cells were detached by trypsinization of the cells. Cells were washed with PBS and resuspended in lysis buffer (30 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM MgCl₂, 1% CHAPS). Immunoprecipitation was performed using Dynabeads® M-280 Tosylactivated (Invitrogen) conjugated with rPru p 3 and LTP1 and the lysate from Caco 2 cells, following manufacturer's instructions.

The Dynabeads® M-280 Tosylactivated was loaded in an SDS-PAGE gel and stained with silver, following manufacturer's instructions (Bio-Rad Laboratories) to visualize the retained proteins. Protein identification was performed by nLC-MS/MS spectrometry (ion trap).

Immunolocalization of Pru p 3 in Caco 2 monolayer culture

Pru p 3 labelled with Alexa 550 using Alexa Fluor® 550 Protein Labelling kit (Molecular Probes, Oregon USA) was cultured with a polarized Caco 2 cells. After 40 min, cells were washed with PBS and fixed in 4%

formaldehyde 4°C for 15 min. After washing with PBS, cells were incubated with Vibrant Lipid Raft labelling Alexa 488 (Molecular Probes, Eugene, OR, USA), following the manufacturer's instructions. Samples were mounted with glycerol : PBS (1 : 1) and observed on a Leica TCS-SP8 confocal microscope, under the laser excitation lines of 488 and 561 nm. Collection of stacks was optimized to the maximum Z resolution.

Gene expression by qPCR

The expression of cytokines was determined by real-time PCR. mRNA was isolated according to the Qiagen-RNeasy protocol (Qiagen, Valencia, CA, USA) and stored at -80°C. RT-PCR was performed as previously described [22]. cDNA was amplified using the Power SYBR Green PCR Master Mix (Applied Biosystems, Alcobendas, Spain) according to the manufacturer's recommendations and run on an Applied Biosystems 7300 real-time detection system (Applied Biosystem), using previously described primers [30–32]. The amount of the target mRNA expression was normalized with endogenous control EF-1 [33], and relative quantification was performed using the comparative threshold cycle method ($2^{-\Delta\Delta C_t}$), as described by Livak and Schmittgen [34]. The changes in gene expression were calculated with respect to the untreated cells. All amplifications were carried out in triplicate, and represented results were derived from four different assays.

Statistical analysis

Data were expressed as mean \pm SDs of four independent experiments. Statistical analysis was performed using SPSS 17.0 (IBM, Chicago, IL, USA) and Statgraphics Centurion XVI. Pru p 3 and LTP1 were compared using Wilcoxon paired samples test or Mann–Whitney. A level of significance of <5% ($P < 0.05$) was considered to be significant in all analyses.

Results

Pru p 3 can cross the Caco 2 cell monolayer not mediated by proteolytic activity

Before the allergen arrival to intestinal epithelium, it is subjected to gastrointestinal digestion by proteolytic enzymes present in the stomach and in the small intestine. However, the simulation of gastrointestinal digestion of Pru p 3 (SGF, Simulated gastric simulation; GIT, Gastrointestinal treatment; Fig. 1a) involved no change. Therefore, it was decided to use the untreated protein in the study of the transport of Caco 2-monolayer.

To measure the transport of Pru p 3 across the Caco 2 monolayer, cells were grown on Transwell® inserts,

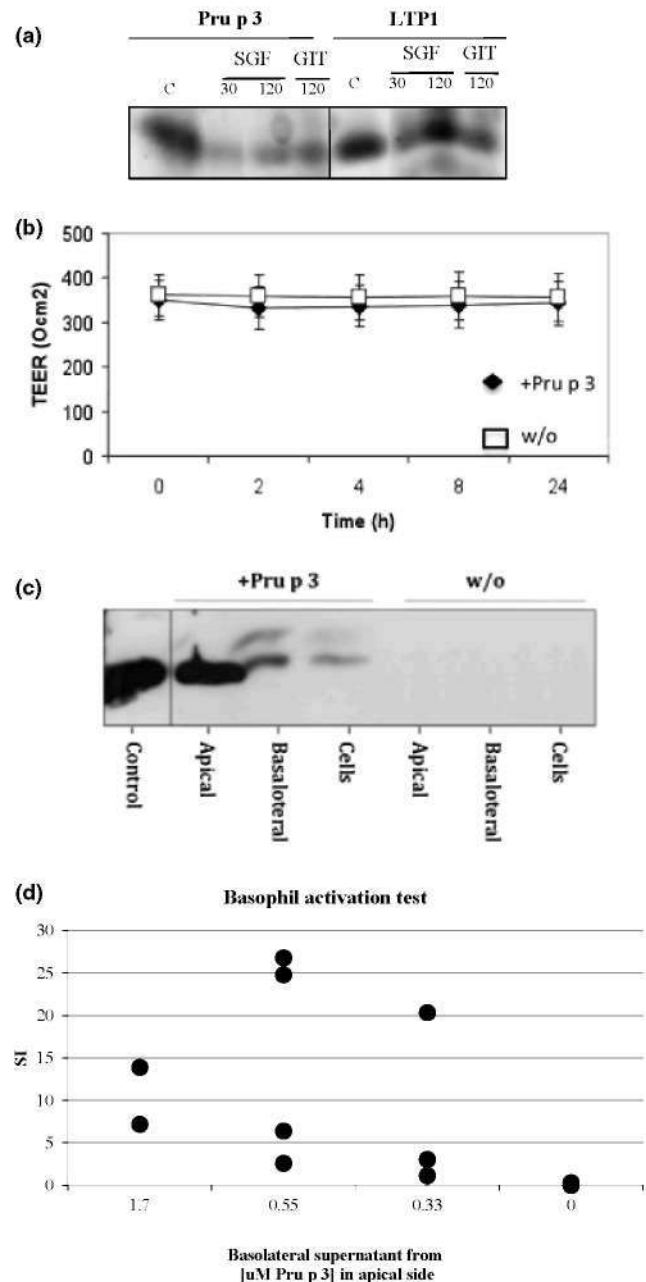


Fig. 1. Transport of Pru p 3 across Caco 2 cell monolayer. (a) SDS-PAGE of Pru p 3 and LTP1 after simulation gastrointestinal digestion. Proteins were incubated with pepsin in HCl 100 mM during 120 min (SGF, 30', 120' after pepsin adding). After inhibition of pepsin activity, gastrointestinal treatment was carried out during 120 min (GIT, 120'). (b) TEER evolution during 24 h of incubation with Pru p 3 and control cells without antigen. TEER values correspond to the mean of four independent experiments. Means and SD (bars) are shown. (c) Western blot using anti-Pru p 3 antibody. Caco 2 cells incubated with Pru p 3 (5 μg) for 24 h and control cells without allergen were recovered and loaded on SDS-PAGE. Control, Pru p 3 positive control; apical, apical supernatant; and basolateral, basolateral supernatant; cells, cell fraction. (d) Basophil activation test with supernatants from Caco 2 Transwell® inserts, incubated with Pru p 3, with whole blood of a peach-allergic patient. Activated basophils% (SI) to supernatants with different concentrations of Pru p 3 in the apical side.

for 18–21 days, until the monolayer was fully differentiated and the tight junctions correctly formed (TEER $>300 \Omega\text{Wcm}^2$).

The allergen was added to the apical side and was incubated with Caco 2 cells for 24 h. During this time, TEER was measured to check the integrity of the monolayer (Fig. 1b). The values with and without Pru p 3 were not statistically significant (Mann–Whitney *U*-test, $P = 0.634$), indicating that the allergen did not affect the integrity of the monolayer. The cell fraction and supernatants from the apical and the basolateral sides were recovered after the culture with Pru p 3, and an immunoblot assay was performed using a specific polyclonal antibody (Fig. 1c). The allergen was detected not only in the apical chamber but also in the basolateral medium and in the cell fraction. Pru p 3 was observed in both fractions as double bands.

The allergenic activity of Pru p 3 present in the basolateral supernatant was also tested by means of basophil activation tests (BAT) (Fig. 1d). After 4 h, there was enough Pru p 3 to produce the cross-linking of IgE in the basophil membrane from a peach-allergic patient, even with an initial amount of 1 μg in the apical chamber (0.33 μM ; Fig. 1d), indicating the conservation of their conformational B-epitopes.

The transport mechanism was more efficient with Pru p 3 than with a non-allergenic LTP

The transport of Pru p 3 labelled with Alexa 488 was measured at different concentrations, in the recovered basolateral medium at several time points (Fig. 2a). After 2 h of incubation, significant amounts of Pru p 3 were measured in the basolateral compartment, and the transport was maintained over the whole 24-h period studied. At the end of the experiment, around 1 μg of Pru p 3 was transported to the other side.

To determine whether the high rate of transport of Pru p 3 across the epithelium was the consequence of an intrinsic characteristic of this allergen or a general phenomenon for the LTP family, the transportation assay was repeated using a natural hypoallergenic LTP from peach, LTP1, which shares approximately 50% amino acid identity with Pru p 3. This protein was purified from the supernatants of *Pichia pastoris* yeast (Fig. 2b), and its reduced allergenic activity was tested by ELISA assay (Fig. 2c). This protein remained intact after being subjected to gastrointestinal treatment (Fig. 1a).

Transport experiments were also performed with LTP1, labelled with Alexa 488, in the same way as with Pru p 3. The rate of transport was much lower in the case of the hypoallergenic LTP (Mann–Whitney test, $P = 0.03$), indicating that Pru p 3-transport occurs via a specific mechanism that may not apply to all LTPs (Fig. 2a).

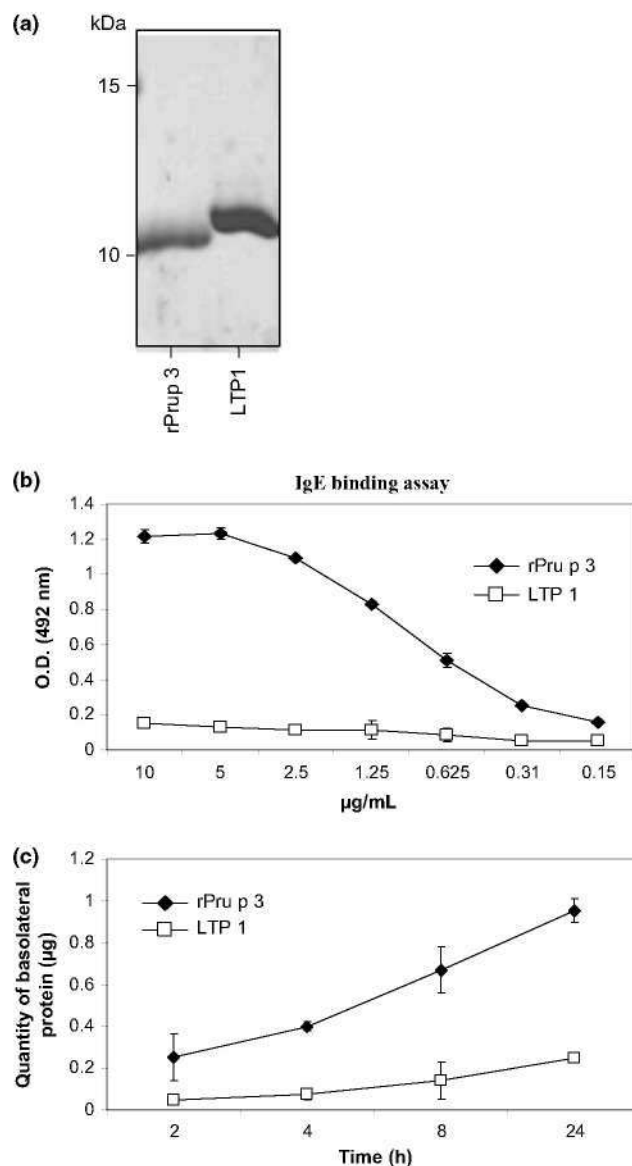


Fig. 2. Comparison of the activity of Pru p 3 vs. the non-allergenic peach, LTP1. (a) Pru p 3 and LTP1 (3 μg) were separated by SDS-PAGE and stained with Coomassie Blue. (b) Specific IgE to Pru p 3 and LTP1 (3 $\mu\text{g/mL}$), using a pool of sera from peach-allergic patients (1 : 2 dilution). Blocking solution without solid phase was used as a negative control, and optical density (OD) values > 0.189 units ($n = 3$; mean $\pm 3 \times \text{SD} = 0.108 \pm 3 \times 0.026$ OD units) were considered positive. All tests were performed in triplicate. (c) Pru p 3 and LTP1 (5 μg) labelled with Alexa 488 were added to the apical side, and the basolateral medium was recovered at different times. Cumulative transport of the proteins in the basolateral medium was measured by fluorescence. The results correspond to four independent experiments. Means and SD (bars) are shown.

Pru p 3 transport was mediated by proteins related to vesicular transport

The curve of transport rate of the allergen as a function of the initial amount in the apical side was close to

hyperbolic and followed the Michaelis–Menten equation which is characteristic of a receptor-mediated transport [35] (Fig. 3a). To try to determine the putative transporter of Pru p 3 and LTP1, an immunoprecipitation experiment was performed, using dynabeads conjugated with both proteins and Caco 2 cell lysate. Following immunoprecipitation, a main band was visible on SDS-PAGE by silver staining (Fig. 3b). The band fingerprint matched with some proteins, all related to the vesicle transportation system (Table 1). The closest match for Pru p 3 was obtained for junction plakoglobin (P14923).

Similarly, the band obtained for LTP1 matched with proteins related to the vesicle transport system (Table 1), although this case, the most expected was obtained with the polymeric immunoglobulin receptor (PIGR, P01833).

In addition, Caco 2 polarized cells incubated with the allergen labelled with Alexa 550 were observed at the

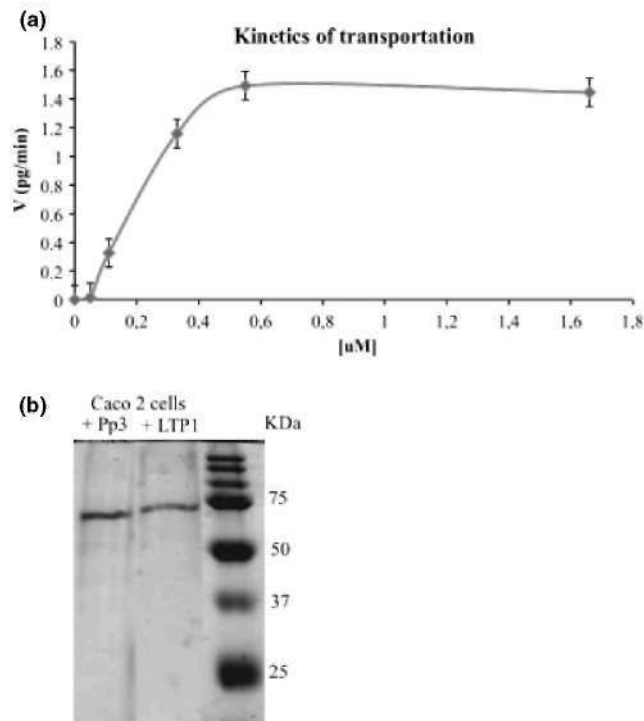


Fig. 3. (a) Curve of transport kinetic of Pru p 3 as a function of initial amount in the apical side. Pru p 3 labelled with Alexa 488 was added to the apical side, and the basolateral medium was recovered at different time points. Cumulative transport of the allergen in the basolateral medium was measured by fluorescence. The results correspond to four independent experiments. Means and SD are shown. (b) Immunoprecipitation assays with Pru p 3 and LTP1 bound to Dynabeads® M-280 Tosylactivated and Caco 2 cell lysated. Immunoprecipitated fraction was loaded on SDS-PAGE and stained with Coomassie Blue R 250. The identification of bands was carried out by finger printing and mass spectrometry.

Table 1. Pru p 3 and LTP1 associated proteins identified by immunoprecipitation assays. The identification was made by Mascot database

| | Accession | Description | Score | Coverage |
|-----------|---------------|---|--------|----------|
| IP Pru p3 | P14923* | Junction plakoglobin OS = Homo sapiens | 125.90 | 7.89 |
| | Q92841* | Probable ATP-dependent RNA helicase DDX17 OS = Homo sapiens | 88.37 | 2.95 |
| | Q9NZI8* | Insulin-like growth factor 2 mRNA-binding protein 1 OS = Homo sapiens | 87.60 | 3.69 |
| | P04406 | Glyceraldehyde- 3-phosphate dehydrogenase OS = Homo sapiens | 66.59 | 3.64 |
| | P14866 | Heterogeneous nuclear ribonucleoprotein L OS = Homo sapiens | 58.00 | 4.78 |
| | Q02878 | 60S ribosomal protein L6 OS = Homo sapiens | 56.65 | 4.75 |
| | gi:456345* | Polymeric immunoglobulin receptor [Homo sapiens] | 40.00 | 8.00 |
| | gi:264657419* | Immunoglobulin heavy chain variable region [Homo sapiens] | 34.00 | 12.00 |
| IP LTP1 | gi:72535486* | Immunoglobulin kappa CDR3 region, partial [Homo sapiens] | 41 | 7.00 |
| | gi:119601844 | Tetratricopeptide repeat domain 7B, isoform CRA_f [Homo sapiens] | 83.00 | 10.00 |
| | gi:222418791* | EF-hand calcium- binding domain- containing protein 4B isoform a [Homo sapiens] | 32.00 | 7.00 |
| | gi:16416136* | RPGR-interacting protein 1 [Homo sapiens] | 31.00 | 6.00 |
| | | | | |

*Proteins found in lipid rafts and caveolae.

confocal microscope (Fig. 4). Pru p 3 was observed to localize with regions of the membrane identified as lipids rafts by specific staining.

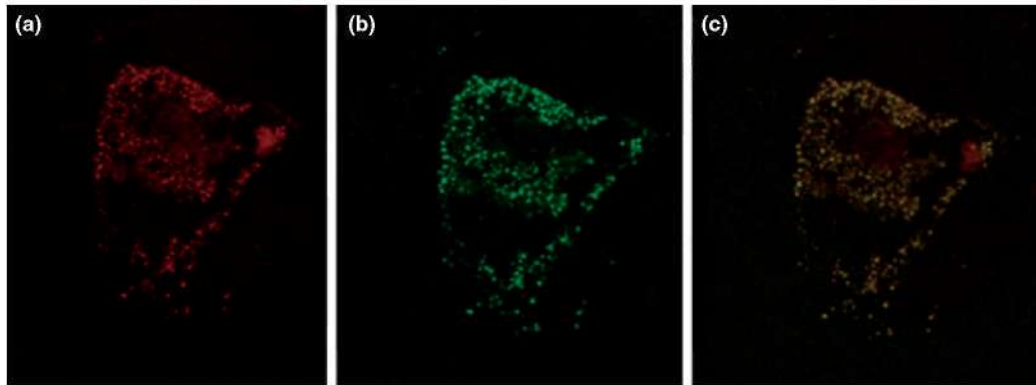


Fig. 4. Immunolocalization of Pru p 3 Labelled to Alexa 550 (Red) and Lipid rafts (Alexa 488, Green) in polarized Caco 2 monolayer after 40 min 11 of incubation. (a) Lipid rafts-Alexa 488 Labelled. (b) Pru p 3-Alexa 550. (c) Merged.

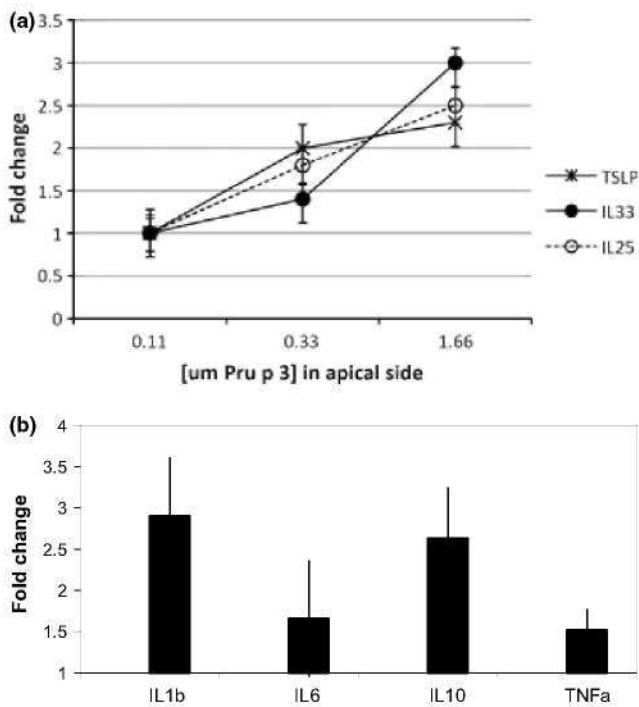


Fig. 5. Cytokine expression measured by qPCR. Pru p 3 (5 μ g) was incubated in the apical side for 12 h. (a) TSLP, IL33 and IL25 relative gene expression levels for Caco 2 cell monolayer. (b) Cytokine gene expression produced by PMBCs in coculture with Caco 2 monolayer, recovered from basolateral side. The results are expressed in fold change referred to wells without antigen. All amplifications were performed in triplicate and four independent assays. Means and SD (bars) are shown.

Pru p 3 maintains its allergenic capacity after crossing the cellular monolayer by inducing Th2 cytokine production

The effect of Pru p 3 in epithelial cells was investigated by quantifying the expression of the epithelial cytokines TSLP, IL33, and IL25 at RNA level by qPCR

(Fig. 5a). The expression of these cytokines was normalized with the non-stimulated control and represented as fold change. An increase in the expression of such cytokines was observed concentration dose dependent of allergen present in the apical side.

In the same way, cytokine expression was quantified in coculture with PMBCs from healthy donors in the basolateral chamber of Transwell[®] inserts. Although there was great variation in terms of the levels of cytokine produced in different donors, PMBCs produced mainly IL1b, IL6, IL10, and TNF α (Fig. 5b).

Discussion

In this study, Caco 2 cells were used as a simplified model of intestinal epithelium to study the transport of Pru p 3 to the basolateral side, using Transwell[®] inserts. The presence of the allergen did not affect the monolayer culture. Pru p 3 passed through the monolayer via a non-proteolytic mechanism, as described for other plant allergens (Bet v 1, Phl p 1) [9, 10].

In this epithelial model, there are two possible routes for the transport through epithelium: the paracellular route (between cells) and the transcellular route (in vesicles) [36]. In the case of Pru p 3, the TEER was maintained until the end of the experiment, indicating that the tight junctions were well formed and were not affected by the allergen. This implies that paracellular transport did not take place and suggests that the peach allergen may follow a transcellular route, which has been previously suggested for other members of the same family, as in the case of the wheat LTP, Tri a 14 [12], and other food allergens such as bovine milk allergens, β -lactoglobulin, and α -lactalbumin [37, 38]. The paracellular route was confirmed by confocal microscopy, in which Pru p 3 colocalized with lipid rafts.

When dietary proteins arrive at the gut, they are subjected to selective transport. This selection prevents dietary antigens and microbes from crossing the barrier,

and it is a highly regulated process [36, 39]. The selective transportation has two functional routes: a major lysosomal degradative pathway and a minor non-degradative pathway. In the case of Pru p 3, its transport may be related to its high resistance to endolysosomal degradation [40, 41] or to interaction with some cell components justifying the double band observed in immunoblot.

In addition, the Pru p 3 transport seemed to follow a Michaelis–Menten kinetic, which pointed to a receptor-mediated transport [35]. A putative receptor was identified by immunoprecipitation assays matching with a protein homologous to junction plakoglobin [42] (γ -catenin). It has been reported that Bet v 1, the major allergen of birch pollen, was taken up through lipid rafts and caveolae into epithelial cells in allergic patients [9]. In fact, catenins have been described as a link between adherent junctions and caveolae-mediated vesicular transport. In addition, β -catenin has been found to have a role in Th2-type response promoting GATA3 expression [43].

The high transport rate of Pru p 3 and the interaction with the vesicular transportation system seemed to be related to its allergenic activity, due to the fact that the transport of the non-allergenic peach LTP1 was much lower, only reaching the basolateral chamber residual amounts [44].

After crossed the monolayer, Pru p 3 was able to activate basophils from a peach-allergic patient, indicating that the allergen reaching the basolateral chamber of Transwell® inserts maintained its integrity, or at least the conformational B-epitopes that are recognized by the IgE in basophil membrane. Previous studies [44–46] have reported the capacity of enterocytes to internalize and transport small amounts of intact proteins or large fragments with antibody binding capacity involved in the immunologic sensitization to food allergens. Pru p 3 induced the production of Th2 cytokines by the epithelial cells. An induction of TSLP, IL25, and IL33, related to Th2 response [2, 47–52], was measured after culturing Caco 2 monolayer with the allergen. The capacity of basolateral Pru p 3 to induce an immune

response was also confirmed in Caco 2-PBMCs cell cocultures. The expression of inflammatory cytokines such as IL1b, IL6, and IL10 by PBMCs was observed as previously described [53]. The highest induction with respect to the unstimulated cells was for IL1b, which has been related with a Th2 response and antibody production in several studies [54–56] suggesting a possible role in Pru p 3 sensitization. The cytokine expression observed with LTP1 was significantly lower, which may be a consequence of the lower transport rate (data not shown).

In conclusion, Pru p 3 was transported in an intact form by intestinal epithelial Caco 2 cells and had the capacity to induce an immune response in underlying cells. Pru p 3 seemed to interact with a specific component of desmosomes. The high rate of Pru p 3 transport may be responsible for its sensitizing capacity. Although other mechanisms are involved in a complex epithelium, receptor-mediated transport seems to be implicated in the transport of this allergen. This did not rule out the possibility that transport could be increased by other means, for example, by dendritic cells [57] or by specific immunoglobulins [7, 58].

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Conflict of interest

The authors declare no conflict of interest.

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